DEVELOPMENTAL BIOLOGY **186,** 185–201 (1997)
ARTICLE NO. DB978594

Intracellular Injections of a Soluble Sperm Factor Trigger Calcium Oscillations and Meiotic Maturation in Unfertilized Oocytes of a Marine Worm

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How sperm trigger activating calcium transients in eggs remains a central, unresolved question in fertilization biology. To determine if a soluble sperm factor can generate a fertilization-like calcium response in the absence of sperm-egg binding, aqueous extracts of sperm from the nemertean worm *Cerebratulus lacteus* were mixed with Ca^{2+}-sensitive fluorescent dyes and injected into unfertilized, metaphase-I-arrested oocytes. Based on confocal imaging analyses, unfertilized oocytes that had been injected with sperm extract routinely produced oscillating Ca^{2+} waves and resumed meiotic maturation in a manner that closely resembled normal fertilization. Calcium oscillations and maturation were typically lacking in control oocytes that had been (i) injected with buffer alone or with buffer containing added calcium, (ii) given external treatments of the sperm factor, or (iii) injected with extracts made from cells other than sperm. Boiling or protease treatment essentially abolished the potency of the sperm extract, and nonboiled extracts retained full activity in >10-kDa fractions, but not in <10-kDa fractions. Collectively, such findings suggest that the sperm of *C. lacteus* possess a soluble protein that can bypass oolemmal surface receptors to act within the ooplasm as a trigger of repetitive Ca^{2+} waves and meiotic maturation. Results obtained in this study are discussed with respect to the minimum amount of extract needed for egg activation and whether the oscillogenic substance is sufficiently concentrated in a single sperm to play a biological role during fertilization.

INTRODUCTION

During fertilization, sperm elicit a transient rise in the concentration of intracellular calcium ions within eggs (Whitaker and Swann, 1993; Shen, 1995; Kline, 1996). Such fertilization-induced Ca^{2+} transients appear to be universally required for animal development (Nuccitelli, 1991) and consist of either a single, relatively long-lived ''Ca^{2+} wave'' (Gilkey *et al.*, 1978; Nuccitelli *et al.*, 1993; Gillot and Whitaker, 1994; Stricker *et al.*, 1994) or a series of rapidly decaying transients, called ''Ca^{2+} oscillations'' (Miyazaki *et al.*, 1993; Deguchi and Osanai, 1994; Fissore and Robl, 1994; McDougall and Sardet, 1995).

Despite intensive analyses, the exact mechanisms by which sperm trigger a calcium response in eggs remain controversial (Dale, 1988; Whitaker and Swann, 1993; Schultz and Kopf, 1995; Jones and Whittingham, 1996), and essentially two kinds of hypotheses regarding fertilization-induced Ca^{2+} signaling have been proposed. According to ''contact-type'' hypotheses, the sperm attaches to extracellularly exposed receptors on the oolemma of the egg, and such ligand–receptor binding activates G-proteins and/or tyrosine kinases associated with the egg membrane (Jaffe, 1990; Foltz and Shilling, 1993; Carroll and Jaffe, 1995; Williams *et al.*, 1996). Activation of these molecules can in turn trigger Ca^{2+} transients via an intracellular signaling cascade (Fig. 1A).

''Content-type'' hypotheses, on the other hand, propose that sperm introduce soluble substances inside the egg following gamete fusion and thereby induce Ca^{2+} transients without necessarily utilizing signals generated at the egg surface. Dale *et al.* (1985) originally demonstrated that sea urchin eggs are at least partially activated by intracellular injections of sperm extracts, and since then evidence has been obtained that such internally acting sperm factors might be (i) calcium ions themselves, as postulated in ''calcium bomb'' hypotheses (Créton and Jaffe, 1995); (ii) other small molecules, such as inositol 1,4,5-trisphosphate (IP_3) (Iwasa *et al.*, 1990); or (iii) a soluble protein, as maintained in the ''sperm oscillogen'' hypothesis of signal transduction (Swann, 1993, 1996) (Figs. 1B and 1C).

Although such views of fertilization have supporting

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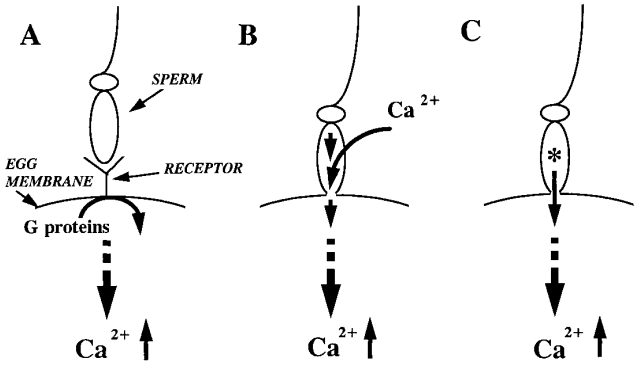

FIG. 1. Sperm contact vs sperm content hypotheses of egg activation. (A) Contact-type hypotheses postulate that sperm bind to extracellularly exposed receptors on egg membranes to activate G-proteins and/or tyrosine kinases, which in turn stimulate an intracellular cascade resulting in Ca^{2+} transients and egg activation. (B and C) Content-type hypotheses propose that following gamete fusion, the sperm introduces soluble substances inside the egg to trigger Ca^{2+} transients and egg activation. Such internally acting substances can be small molecules such as the calcium ions proposed in calcium bomb hypotheses (B), or a soluble protein (*), as maintained in the sperm oscillogen hypothesis of signal transduction (C).

data, findings that are inconsistent with these hypotheses can also be cited. For example, sperm-binding receptors occur on the oolemma (Foltz, 1995), and unfertilized eggs are capable of generating Ca^{2+} transients via intracellular cascades that involve G-proteins and/or tyrosine kinases (Shilling *et al.*, 1994). However, there is little direct proof that the binding of sperm to oolemmal receptors is actually sufficient or necessary for producing activating Ca^{2+} transients during normal fertilization. External applications of an egg-binding sperm peptide activate *Xenopus* oocytes (Shilling *et al.*, 1996), but complete activation of mammalian eggs by such sperm-derived molecules has not been clearly demonstrated (Swann, 1996). Moreover, Ca^{2+} transients and normal development still occur when oolemmal receptors are bypassed by intracytoplasmic sperm injections (Swann *et al.*, 1994), suggesting that such surface receptors are not universally required for the Ca^{2+} response and perhaps play alternative roles during fertilization. Accordingly, the intracellular signaling machinery that can be stimulated downstream to these receptors in laboratory trials (Kline *et al.*, 1991; Moore *et al.*, 1993) may remain dormant during fertilization and only function at postfertilization stages of development in some species.

Calcium bomb hypotheses are also weakened by the fact that the injection or electroporation of calcium into unfertilized eggs often fails to mimic the full Ca^{2+} response of fertilization, particularly in species displaying Ca^{2+} oscillations (Swann and Ozil, 1994). Similarly, although evidence has been obtained in mammals to support the sperm oscillogen hypothesis (Parrington *et al.*, 1996; Swann, 1996; Wu *et al.*, 1997), such supportive data are apparently lacking for nonmammalian species, as the putative sperm factors of sea urchins (Osawa, 1994) and amphibians (Iwao *et al.*, 1995) tend to be (i) relatively small, heat-resistant molecules; (ii) active when applied outside the oolemma; and/or (iii) incapable of triggering the entire Ca^{2+} response in unfertilized eggs. To clarify these issues, additional analyses are warranted, especially in alternative model systems that can supplement the database which has been derived mainly from studies on mammals, frogs, and sea urchins.

The marine worm *Cerebratulus lacteus* has separate sexes and resembles most other members of the phylum Nemertea by undergoing external fertilization without copulation (Stricker, 1987). In response to fertilization, oocytes of *C. lacteus* generate an oscillating series of intracellular Ca^{2+} waves and complete meiotic maturation (Stricker, 1996). Calcium oscillations also occur when whole sperm are injected into unfertilized oocytes, but such oscillations are eliminated if sperm are boiled prior to injection, suggesting the possible presence of a heat-labile sperm factor in *C. lacteus* (Stricker, 1996).

In this investigation, calcium-sensitive fluorescent dyes and confocal microscopy are used to assess the effects of injecting an aqueous extract of sperm into unfertilized *C. lacteus* oocytes. Such injections avoid interactions between whole sperm and oolemmal receptors but nevertheless cause repetitive Ca^{2+} waves with spatiotemporal properties closely resembling those observed during normal fertilization. The Ca^{2+} oscillations are routinely accompanied by meiotic maturation, and neither Ca^{2+} oscillations nor maturation tend to occur in control oocytes that were (i) given external treatments of the sperm factor or (ii) injected with either calcium-containing buffers or extracts made from cells other than sperm. The sperm extract is heat- or protease-labile, and its biological activity is fully retained in >10-kDa fractions, but not in <10-kDa fractions. Collectively, these findings provide new evidence in a nonmammalian species for a soluble sperm protein that can elicit Ca^{2+} oscillations from within the ooplasm. The data also document for the first time that intracellular injections of sperm extract can consistently trigger meiotic maturation. Whether or not the putative sperm factor is sufficiently concentrated in a single sperm to activate eggs is discussed based on semiquantitative calculations and the results obtained from injecting single whole sperm.

MATERIALS AND METHODS

Animals and Gametes

Sexually mature specimens of *C. lacteus* were purchased from the Marine Biology Laboratory, Woods Hole, MA, and kept at 12–14°C within floating baskets in aerated aquaria filled with ''Instant Ocean'' artificial seawater (ASW). Uncompressed, prophase-arrested oocytes that were obtained from cut pieces of ripe females typically ranged in diameter from 100 to 130 μm (Stricker, 1996) and were stripped of their jelly coats by repeated passages through a 150-μm-mesh Nitex filter. After incubating 2 hr in ''MBL'' ASW (Cavanaugh, 1956), most oocytes completed germinal vesicle break-

down and became arrested at metaphase I of meiosis in preparation for fertilization (Stricker, 1996). Only oocytes that had been isolated from the ovaries for 2–8 hr were used in the experiments, since the percentages of normal development to the blastula stage dropped from maximal rates of ~55–90% in laboratory cultures to <5%, if aged oocytes were inseminated >8 hr postisolation.

Microinjections and Confocal Imaging of Calcium Dynamics

Washed, metaphase-I-arrested oocytes were attached to protamine-coated specimen dishes and injected with an Eppendorf high-pressure system (Stricker, 1996). Since calcium aids in wound healing, all injections were conducted in calcium-containing MBL seawater. However, after some injections, the seawater was replaced with EGTA-containing calcium-free seawater (CaFSW) to test the effects of reducing external Ca^{2+} to <50 n*M* (Stricker, 1996).

To avoid the deleterious effects of double impalements, oocytes were given a single injection of a 1:1 mixture of (i) fluorescent dyes and (ii) either sperm extract or a control solution. The dyes consisted of 10,000 MW dextran conjugates of calcium green (CG) and rhodamine B (Rh) (Molecular Probes, Inc.) at 1.25 and 0.1 m*M*, respectively, in injection buffer (IB), which comprised 10 m*M* Hepes and 100 m*M* potassium aspartate at pH 7.2 (Chiba *et al.*, 1990). For each specimen dish, several oocytes were routinely injected over a period of 3–9 min. Confocal imaging was not initiated until 2–6 min after the last injection was made so that (i) the dishes could be transferred to the confocal system (where injections were not attempted, owing to space constraints); (ii) the injectate was able to diffuse throughout the cell; and (iii) any Ca^{2+} flux caused by the impalement of the injection pipet could decay back toward preinjection levels. Thus, imaging of most specimens began ~5–10 min postinjection, and the oocytes' initial responses to the injection were not investigated.

With the high-pressure injection setup used in this study, precisely quantified injections such as performed using the double-droplet technique (Kishimoto, 1986), are difficult to conduct on multiple oocytes, especially when a biologically active injectate necessitates that imaging starts without a significant delay. Hence, to obtain large enough sample sizes that could be checked by a variety of controls, a simple visual appraisal of each injection volume was made based on the size of the injected bolus, which in turn could be recognized by the orange coloration of the fluorescent dyes. By using a calibrated ocular micrometer to measure the diameter of the injectate relative to that of the oocyte, it was estimated that the injectate approached ~1% of the oocyte volume on a few occasions, but typically constituted ~2–5% of the oocyte volume. As discussed by Stricker *et al.* (1994), the repeatability and general reliabilities of such estimates were verified prior to the onset of this study by *in vitro* calibrations using dye droplets serially diluted in 60 n*M* calcium buffer (Molecular Probes Inc.).

For analyses of calcium dynamics, dye-loaded specimens were subjected to dual-channel confocal imaging using a Bio-Rad MRC-600 confocal system (Stricker, 1996). By simultaneously collecting the calcium-sensitive CG signal and the calcium-insensitive Rh signal, general trends in $[Ca^{2+}]_i$ were monitored every 5 or 15 sec within a ~5-μm-thick optical plane that remained fixed near the center of the oocyte. Such dual-channel images were then corrected for offset in each channel (Finkbeiner, 1992), ratioed, and graphed over time in a normalized form as $R - R_0/R_0$, where R_0 is the initial CG/Rh ratio, R is subsequent CG/Rh ratios, and a value of 1 represented a doubling of the CG/Rh ratio. Based on previous calibrations (Stricker, 1996), fertilization-induced changes in the CG/Rh ratio typically correspond to $[Ca^{2+}]_i$ fluxes on the order of several hundred nanomolar.

The possibility that changing the focal plane's position within the oocyte might introduce an artifact in the CG/Rh ratioing procedure has not been quantitatively analyzed. However, the optical plane was set near the center of the oocyte throughout the imaging run, and Ca^{2+} oscillations propagated across the entire optical section without any noticeable change in cell shape that might alter the depth of the focal plane within the oocyte (Stricker, 1996). Such imaging conditions in turn argue against the notion that depth-related artifacts in the CG vs Rh signals significantly affected the results recorded in this study.

Ratioed images were routinely converted into pseudocolored montages of individual Ca^{2+} transients. Blue regions of such montages corresponded to relatively low CG/Rh ratios and $[Ca^{2+}]_i$, whereas red areas encoded higher ratios and calcium levels (Stricker, 1995, 1996). Alternatively, the pseudocolored circular images of each oocyte were stacked together to form a ''3-D cylinder'' whose vertical axis represented time, and the cylinder was then cleaved longitudinally to display Ca^{2+} dynamics throughout the time-lapse run (Stricker, 1994).

Following imaging, specimens were scored as undergoing Ca^{2+} oscillations, if two or more transients occurred within 30 min postinjection, and, to avoid including noise, only transients with peaks at least 15% greater than the baseline were counted. The amplitude, duration, frequency, and rate of rise of oscillations were measured as described by Stricker (1996), and statistical analyses involved Student's t or Mann–Whitney U tests (Sokal and Rohlf, 1973).

Sperm Extracts and Control Treatments

To obtain soluble sperm components, ripe males were cut in a beaker containing 20 ml of IB, and 2–4 drops of concentrated sperm emanating from the cuts were added to 1 ml of IB. The sperm sample was then centrifuged for 1 min at 14,000*g* in a 4°C incubator and rapidly washed three times with 1 ml of IB to yield $1–2 \times 10^9$ sperm/ml densities. Such concentrations were determined by hemacytometer readings of the final suspension and were adopted so as to approximate the $0.8–1.5 \times 10^9$ sperm/ml range utilized for mammalian sperm extracts (Homa and Swann, 1994; Dozortsev *et al.*, 1995). Washed samples were frozen in liquid nitrogen and subsequently thawed at room temperature for 30 min before being stirred with a vortex mixer for 1 min and given one or two centrifugations at 14,000*g* for 15 min each in the 4°C incubator. The supernatant from such preparations was devoid of cellular material when viewed in a microscope at 1000× and thus constituted the ''crude sperm extract.''

To fractionate the extract via molecular weight cutoff filters (Amicon Corp.), ~1 ml of crude sperm extract was placed on a 100,000 MW (''C-100'') filter and centrifuged for 20–30 min at 4°C and 6000 rpm in a Beckman GS-6R centrifuge. The retentate was collected as the >100,000-kDa fraction, whereas the filtrate that passed through the C-100 filter was transferred to a 10,000 MW (''C-10'') filter and similarly centrifuged to provide (i) a retentate fraction of 10–100 kDa components and (ii) a filtrate comprising the <10-kDa fraction. Although the protein content of each fraction was not ascertained, the <10-kDa fraction had approximately the same volume of fluid as did the 10- to 100-kD fraction, after being subjected to such relatively short centrifugations. This in turn helped to ensure that any variations observed in these two fractions' activities were not simply due to grossly different concentrating effects of the filters.

For control extracts made from cells other than sperm, washed oocytes were collected at a concentration of about 4×10^4 cells/ml and spun for 10 sec in a centrifuge to produce a pellet that was slightly larger than the sperm pellets used for extracts. The oocyte pellet was then rapidly washed four times in IB, frozen in liquid nitrogen, and subsequently processed according to the methods used on sperm samples to yield a supernatant ''oocyte extract.'' Alternatively, pieces of unripe male worms were frozen in liquid nitrogen and pulverized into a fine powder. About 0.1 g of the frozen powder was then placed in IB and briefly spun in a chilled microcentrifuge to verify that the pellet was one to two times as large as the sperm pellets used for extracts. After resuspension in the IB, the body powder was frozen again in liquid nitrogen and subsequently processed to yield a ''whole body extract'' as described for the sperm extracts.

To determine if the activity of the sperm extract was heat-labile, crude sperm extract was boiled for 10–60 min before being cooled, combined 1:1 with fluorescent dyes, and injected into oocytes. The sensitivity of the sperm extract to protease treatment was analyzed by dissolving 0.15% protease (Type XXIII, Sigma) in IB. Two microliters of the protease was then added to 8 μl of whole sperm extract or the 10- to 100-kDa fraction of the sperm factor to obtain the 0.03% protease concentration used in studies of sea urchin sperm factors (Osawa *et al.*, 1994). The protease-treated sperm extract was incubated at 12°C for 30 min and then either mixed 1:1 with fluorescent dyes and injected into oocytes or treated with 125 μM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Sigma) before being combined with dyes and injected. To ensure that the PMSF treatment by itself did not markedly affect the sperm extract, 8 μl of sperm extract was (i) mixed with 2 μl of IB lacking protease; (ii) incubated at 12°C for 30 min; (iii) treated with 125 μM PMSF; and (iv) mixed 1:1 with dyes, before being injected into oocytes.

External applications of sperm extract were tested by adding non-boiled sperm extract to specimen dishes containing cells that had been injected with fluorescent dyes. For such tests, extract obtained from an $\sim 1.5 \times 10^9$ sperm/ml preparation was mixed 1:19 with the seawater in the dish so that the 5% external concentration was at least twice as high as the 1–2.5% intracellular concentrations attained after a 1:1 mixing with dyes and a subsequent injection corresponding to 2-5% of the oocyte's volume.

Since atomic absorption analyses have shown that sperm extracts contain calcium ions (Dale *et al.*, 1985), attempts were made to determine if excess Ca^{2+} by itself could account for the oscillogenic activity of sperm extracts. For such analyses, 40–50 μM Ca^{2+} was added to injection buffer so that after the high-calcium IB solution was combined with fluorescent dyes and injected into oocytes, ooplasmic $[Ca^{2+}]_i$ would presumably reach the several hundred nanomolar levels normally induced by fertilization. Excess calcium was added either as 50 μM $CaCl_2$ (Homa and Swann, 1994) or as a 10 m*M*, pH 7.2, Ca-EGTA buffer solution containing 40 μM free calcium (Molecular Probes Inc.).

Analyses of Maturation and Development

To analyze meiotic maturation, specimens subjected to confocal imaging were routinely checked for polar bodies at 1–2 hr after injection or fertilization. Only oocytes with polar bodies clearly extending away from the cell circumference were scored as mature, and rates of polar body formation in extract-injected specimens were normalized relative to fertilized controls. About 25–35% of the controls had a recognizable polar body 1–2 hr after fertilization, which in turn represented an 80–100% maturation rate in the specimen dish, given (i) many mature oocytes were not counted as such, because the orientation of the attached cells obscured view of their polar bodies; (ii) in control cultures with 25–35% verified maturation, >80% cleaved; and (iii) cleavage does not occur unless maturation is completed (unpub. obs.).

Polar body formation was also analyzed in specimens that were not subjected to confocal imaging but were instead prelabeled with the DNA-binding probe Hoechst 33342 (Sigma), injected with sperm extract, and viewed by conventional fluorescence microscopy (Stricker, 1996). In addition, the percentage of oocytes with clearly evident polar bodies was quantified in 100–200 oocytes obtained from cultures incubated at 12°C. At the onset of each experiment, the cultures were fertilized or given an external application of either 5% sperm extract or 5% injection buffer. One to two hours later, the incubated cultures were fixed with a formalin/glutaraldehyde solution (Stricker, 1996) and then scored for the resumption of maturation as evidenced by polar body formation.

For many of the controls that did not display Ca^{2+} oscillations during 30–60 min of confocal examination, freshly diluted sperm was added at 10^4-10^5 sperm/ml, and imaging was continued for at least 30 min, in order to check specimen viability by determining whether or not the added sperm elicited oscillations. Specimen dishes that received sperm were subsequently incubated at 12°C, and development was observed at 3–5 hr postfertilization for monospermic cleavage and/or at ~15-20 hr for the presence of normal, prehatching blastulae.

RESULTS

Calcium Oscillations and Meiotic Maturation Regularly Occur in Unfertilized Oocytes of C. lacteus Injected with Sperm Extract, but Not in Various Control Specimens

In response to intracellular injections, 90.5% of the 180 unfertilized oocytes of *C. lacteus* that were injected with crude sperm extract displayed Ca^{2+} oscillations (Figs. 2 and 3A–3E). Sperm extracts that possessed such oscillogenic activity were obtained from five of six ripe males tested, and oocytes from all four females injected with sperm extracts consistently gave a positive calcium response.

Since the microinjection and imaging protocols precluded analyses of Ca^{2+} dynamics directly after injection of the sperm extract, oscillations were first evident ~5–15 min postinjection, and such transients propagated at about 10–15 μm/sec as point-source waves from a repeated onset site in the cortical ooplasm (Figs. 3A–3C). In 17 of 20 specimens whose animal–vegetal axis was subsequently defined by clearly discernable polar bodies, the waves originated in the vegetal hemisphere, indicating the possible presence of a ''vegetal pacemaker'' as described for ascidian zygotes (Speksnijder, 1995).

The repetitive Ca^{2+} waves induced by extract injections closely resembled those elicited by fertilization or the intracellular injection of whole sperm (Table 1). The most variable attribute of the extract-induced waves was frequency. Oscillation frequencies may in turn have depended upon extract concentration, since Ca^{2+} oscillations became infrequent or nonexistent if an extract prepared from a 1–2 $\times$

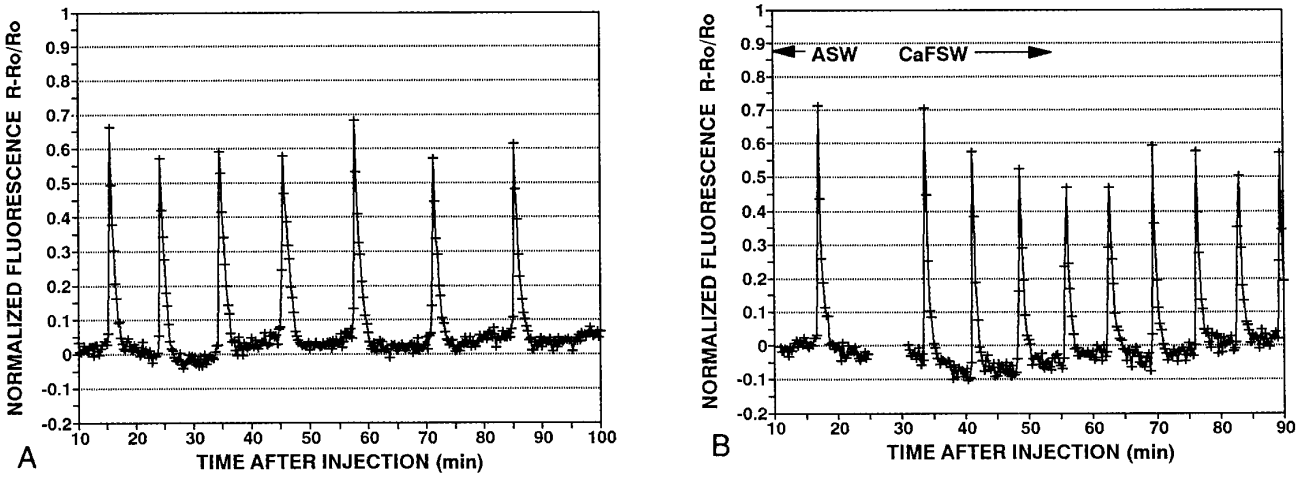

FIG. 2. Ca^{2+} oscillations triggered by sperm extract injections occur in both calcium-containing seawater and in calcium-free seawater. (A) An unfertilized oocyte undergoing oscillations in calcium-containing seawater after injection with sperm extract. (B) Oscillations continue to occur in another sperm-extract-injected oocyte after washing out the calcium-containing artificial seawater (ASW) over a period of 6 min (from 24 to 30 min postinjection) with eight changes of calcium-free seawater (CaFSW), indicating that the repetitive Ca^{2+} waves are maintained by internal calcium release within the oocyte. R_0, initial CG/Rh ratio; R, subsequent CG/Rh ratios.

10^9 sperm/ml sample was diluted 10- or 30-fold, respectively, with IB prior to mixing with fluorescent dyes and injection. It remains to be determined (i) if the oscillations induced by diluted extract had a similar effect on subsequent development as did the oscillations triggered by undiluted extract or (ii) precisely how much the extract could be diluted and still retain full biological activity.

Following sperm extract injections, Ca^{2+} oscillations occurred for 30–120 min and decreased in frequency toward the end of the oscillatory period (Figs. 2A and 3D), as has been observed in fertilized zygotes (Stricker, 1996). Oscillations also persisted in all 10 extract-injected specimens examined after the surrounding seawater was replaced with calcium-free seawater (Figs. 2B, 3B, and 3E).

To ensure that the Ca^{2+} oscillations were due to a specific component of the sperm extract, control oocytes were injected with fluorescent dyes mixed 1:1 with the following IB-based solutions: (i) IB alone; (ii) IB with excess calcium; (iii) extracts made of either oocytes or whole pieces of unripe males; (iv) boiled sperm extract; (v) protease-treated sperm extract; or (vi) the <10-kDa fraction of the extract. Sham controls that were injected with dyes plus IB had a significantly lower percentage of oscillogenic activity at the $P = 0.005$ level than the >90% rate observed in sperm-extract-injected specimens, as 8.1% of the 86 sham controls examined in 14 specimen dishes displayed Ca^{2+} oscillations. Other controls also had significantly lower rates of oscillating activity during the 30-min period after injection than did specimens injected with sperm extract (Table 2; Figs. 3F and 4), and 0 of 12 controls injected with oocyte extract and examined 70–100 min post-injection produced multiple Ca^{2+} waves, indicating that the control treatments did not simply delay oscillation onsets. Not only did "spontaneously oscillating" specimens occur relatively rarely, but their oscillations were in some cases irregular, ephemeral, and/or low in amplitude (Fig. 5) compared to those elicited by fertilization (Stricker, 1996) or sperm extract injections. Thus, the percentages listed in Table 2 conservatively calculate the magnitude of the difference between sperm-extract-injected specimens and controls that fully mimicked a fertilization-like calcium response. The lack of oscillogenic activity was particularly evident among controls that had been injected with IB plus excess calcium. Less than 6% of these oocytes underwent oscillations, as 2 of the 17 specimens given $CaCl_2$, and none of the 17 injected with the Ca-EGTA buffer, displayed multiple Ca^{2+} waves.

The various controls were conducted both before and after sperm extract injections to ensure that the results did not simply reflect some age-related difference in the sensitivity of the oocytes to the treatment. Moreover, many dishes with control specimens were inseminated 45–90 min after injection to determine if nonoscillating specimens were actually capable of producing repetitive Ca^{2+} waves. In 89.7% of the 136 nonoscillating controls tested, Ca^{2+} oscillations were subsequently elicited when sperm was added to the specimen dish (Figs. 4, 5, 10, and 11). The 136 inseminated controls comprised approximately equal numbers from the seven categories listed in Table 2, except for two control groups of questionable viability which were excluded from the dataset: (i) specimens injected with Ca-EGTA buffer, in which only 4 of 17 showed Ca^{2+} oscillations after fertilization and (ii) controls injected with protease-treated sperm extract lacking a subsequent PMSF treatment, where 0 of 12 specimens displayed postinsemination oscillations.

In addition to being linked to Ca^{2+} oscillations, sperm extract injections were routinely associated with the resumption of meiotic maturation (Fig. 3H). In unfertilized specimens injected with sperm extract, polar body forma-

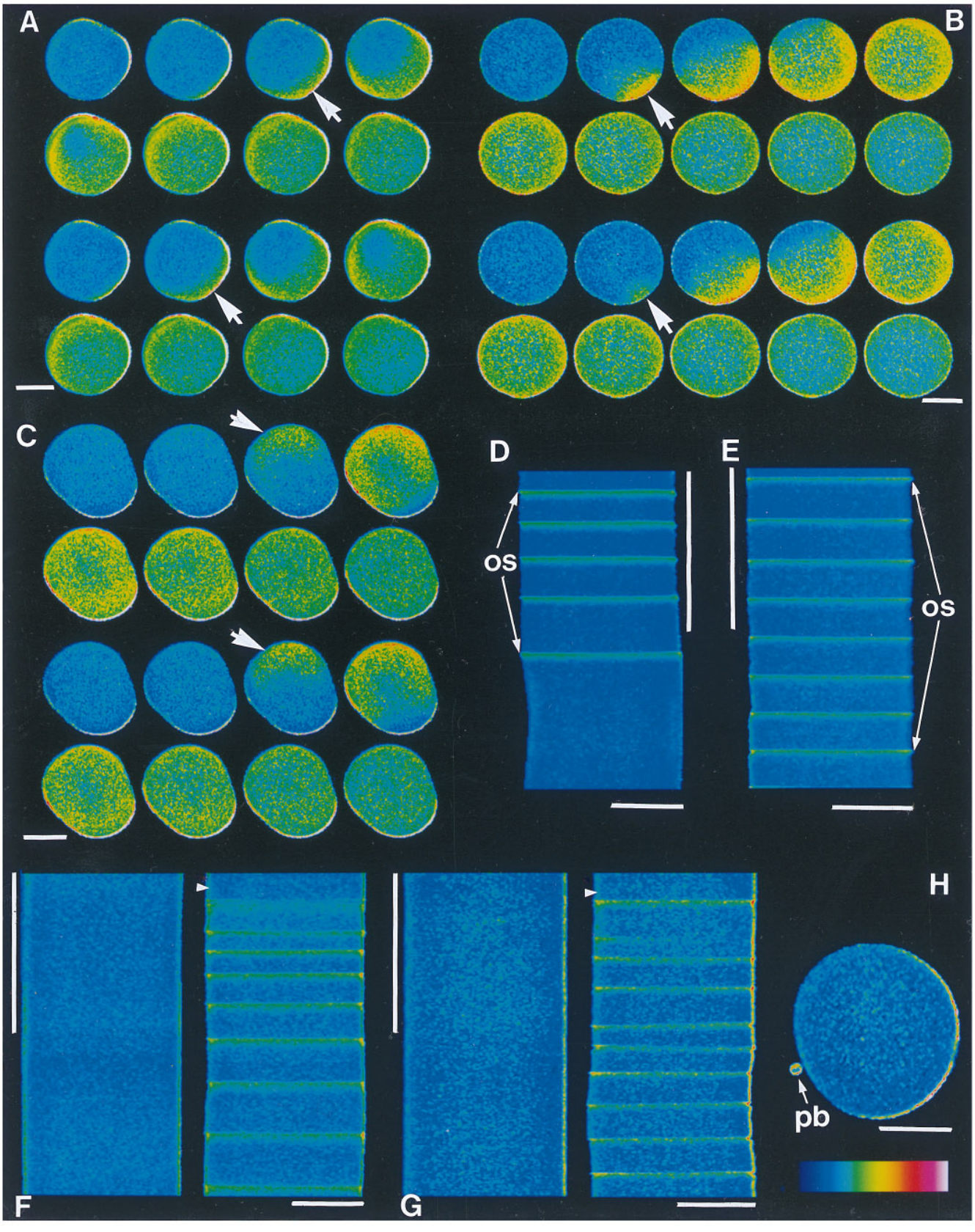

TABLE 1

Ca^{2+} Oscillations Induced by the Injection of Sperm Extract Closely Resemble Those Obtained after Whole-Sperm Injections or Fertilization

	Sperm extract injections	Whole sperm injections[a]	Fertilization[a]
Oscillations propagate as multiple Ca^{2+} waves from repeated onset site in cortex	+	+	+
% Oocytes showing oscillations	90.5%	85.3%	89.7%
(N)[b]	(180)	(41)	(136)
% Oocytes with vegetal pacemaker	85%	78%	79%
(N)[b]	(20)	(9)	(24)
Propagation rate of Ca^{2+} waves (μm/sec)	10–15	10–15	10–20
Amplitude [avg + SD][c]	0.46 ± 0.14	0.49 ± 0.13	0.45 ± 0.18
(N)[d]	(140)	(120)	(150)
Duration (min)[e]	2.8 ± 0.89	3.6 ± 1.6	3.3 ± 1.2
(N)[d]	(140)	(120)	(150)
$T_{1/2}$ rise (sec)[f]	18.1 ± 7.5	19.8 ± 5.4	15.9 ± 7.3
(N)[d]	(100)	(20)	(50)
Time between transients (min)[g]	5.5 ± 2.8	6.7 ± 1.9	4.9 ± 1.8
(N)[d]	(110)	(30)	(77)
Length of oscillatory sequence (min)[h]	75.4 ± 26.5	72.1 ± 7.1	81.6 ± 23.1
(N)[b]	(20)	(10)	(30)

[a] Data from Stricker (1996), except for data from this study on percentage of oocytes showing oscillations after fertilization, and for unpublished observations on (i) propagation rates and frequency of whole sperm injections; (ii) $T_{1/2}$ rise for whole sperm injections and fertilizations, and (iii) length of oscillatory period after whole sperm injections.

[b] Number of oocytes examined.

[c] Peak change in CG/Rh ratio over baseline ratio (R_p-R_b/R_b, where R_p is the peak CG/Rh ratio, R_b is the baseline CG/Rh signal, and a value of 0.46 represents a peak ratio that is 46% higher than the baseline ratio).

[d] Number of Ca^{2+} transients measured.

[e] Duration of a single transient from beginning of transient to return to baseline.

[f] Time required to rise to peak amplitude from halfway between peak and baseline.

[g] Time between successive peaks during the first 30 min after injection or fertilization.

[h] Time of last Ca^{2+} transient after injection or insemination.

tion occurred as frequently as among fertilized oocytes (Fig. 6), and the putative polar bodies induced by extract injections were verified as such by the positive staining observed in specimens loaded with a DNA-binding dye (Figs. 7A and 7B). First polar body formation began ~40–60 min postinjection, and both polar bodies were typically present by ~90

FIG. 3. Dual-channel ratioed images of repetitive calcium waves. In all images, blues represent relatively low CG/Rh ratios and $[Ca^{2+}]_i$, whereas yellows and reds correspond to higher CG/Rh ratios and $[Ca^{2+}]_i$; horizontal scale bars, 50 μm; vertical scale bar, 30 min (the thin bright halo at the edge of some images is an artifact, apparently due to channel misregistration). (A) Two consecutive calcium waves from a repeated onset point (arrowheads) in an unfertilized oocyte injected with sperm extract and imaged at 0.2 Hz in calcium-containing seawater. (B) Two consecutive calcium waves (arrowheads) triggered by injection of sperm extract and imaged at 0.2 Hz after transferring the oocyte to calcium-free seawater. (C) Two consecutive calcium waves (arrowheads) triggered by injection of the 10- to 100-kDa fraction of sperm extract and imaged at 0.2 Hz in calcium-containing seawater; (D–G) Cleaved 3-D cylinders obtained by stacking individual optical sections collected from a single oocyte at 0.07 Hz. The vertical axis represents time, which in turn runs from the top to bottom of each rectangle as imaging proceeds. For (F) and (G), each figure displays a single time-lapse run which has been split into a pair of rectangles for more convenient placement on the page. Imaging begins at the top of the lefthand rectangle and ends at the bottom of the righthand rectangle. (D) Ca^{2+} oscillations (os) induced by injection of sperm extract, showing a dampening in frequency toward the end of the oscillatory period. (E) Continued oscillations (os) after transfer of a sperm-extract-injected oocyte into calcium-free seawater; (F) Before (left) and after (right) fertilization of a control specimen that had been injected with whole body extract, showing no oscillations until sperm is added to the specimen dish (arrowhead). (G) Before (left) and after (right) fertilization of a control specimen that had been injected with fluorescent dyes and given an external treatment of sperm extract, showing no oscillations until sperm is added to the specimen dish (arrowhead). (H) Polar body (pb) present in an unfertilized oocyte at 1 hr after an injection with sperm extract.

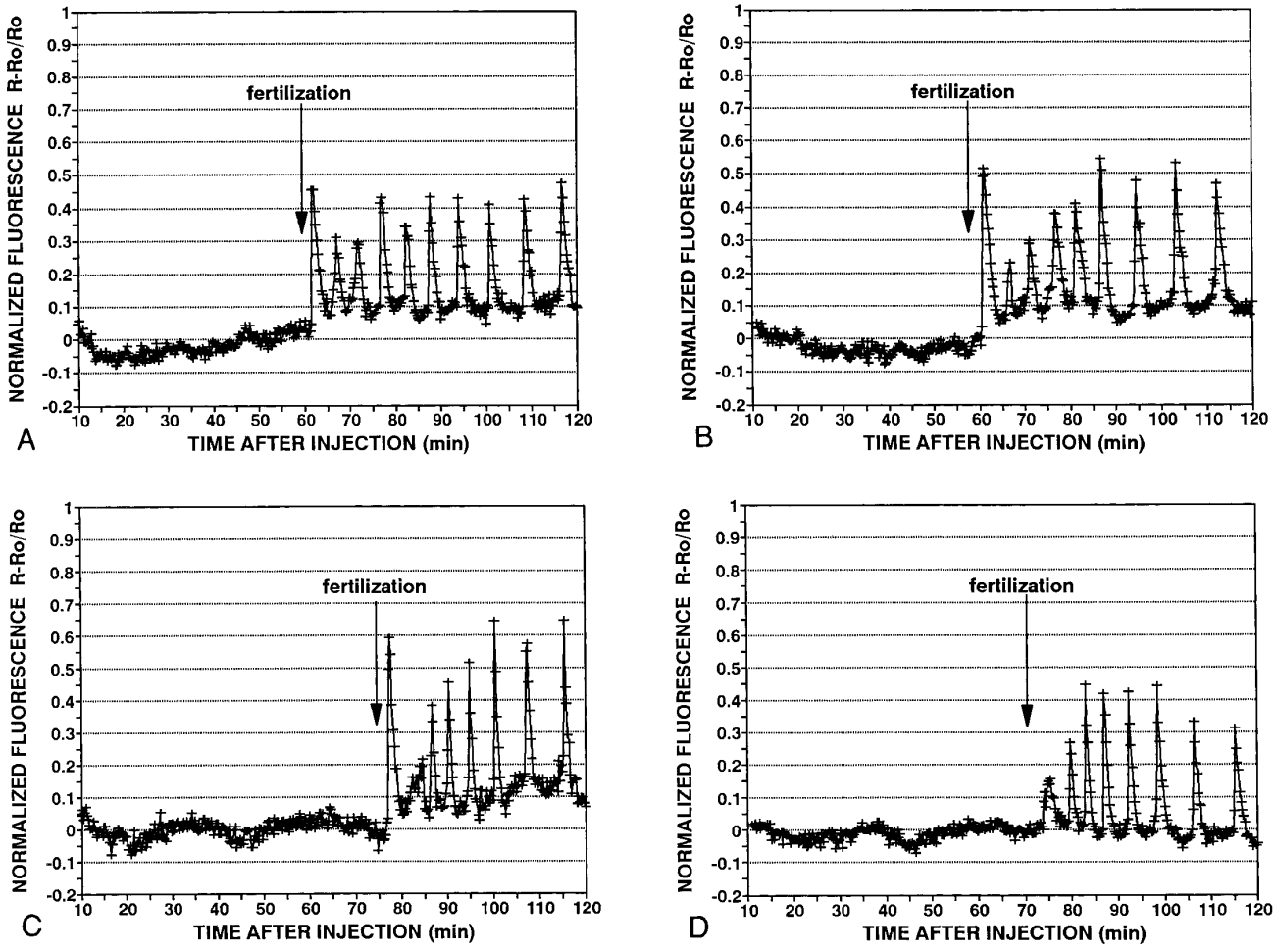

FIG. 4. Various control injections typically fail to elicit Ca^{2+} oscillations, but such controls are capable of undergoing oscillations upon subsequent addition of sperm. Ca^{2+} oscillations are typically lacking in control oocytes during 30–60 min of imaging. However, control treatments do not prevent oocytes from producing multiple Ca^{2+} waves, since oscillations are consistently elicited if sperm is added to the specimen dish at 50–70 min postinjection and fertilization occurs in a previously nonoscillating control that was injected at the onset of the experiment with (A) injection buffer only, (B) injection buffer plus excess calcium (50 μM $CaCl_2$), (C) oocyte extract, or (D) whole body extract. R_0, initial CG/Rh ratio; R, subsequent CG/Rh ratios.

min post-injection, which in turn resembled the timing observed following fertilization (Stricker, 1996). As with the Ca^{2+} oscillations, maturation was essentially lacking in various controls not receiving sperm extract (Fig. 6), and the few aberrant cases of maturation in these oocytes occurred in specimens that previously underwent Ca^{2+} oscillations (unpub. observ.).

In 100 extract-injected oocytes monitored at least 5 hr after calcium imaging, no specimen displaying Ca^{2+} oscillations developed beyond the haploid egg stage and cleaved, suggesting that sperm extracts can induce oscillations and maturation but not full parthenogenesis without the male genetic complement and/or centrosome. Similarly, when oscillating oocytes injected with sperm extract were inseminated about 1 hr after extract injection, the oscillations were not altered or prolonged by the added sperm (Fig. 8), and all 38 of such specimens remained uncleaved 24 hr after sperm addition, suggesting that the sperm extract may elicit a block to subsequent sperm fusion and/or incorporation. Control specimens that were not undergoing Ca^{2+} oscillations prior to fertilization, however, typically responded to inseminations by initiating oscillations and developing into ciliated blastulae within 24 hr (Figs. 7C and 7D).

External Applications of the Sperm Extract Fail to Trigger Calcium Oscillations or Maturation

Aside from control injections, sperm extract was also pipetted into specimen dishes containing oocytes that had been injected with fluorescent dyes and IB. Even though the 5% final concentration of extract in the surrounding seawater at least doubled the 1–2.5% calculated intracellu-

TABLE 2

Ca^{2+} Oscillations Routinely Occur Following Sperm Extract Injections, but Are Rarely Evident after Various Control Injections

Treatment	N^a	% Oocytes with oscillations[b]
Sperm extract injection	180	90.5
Control injections		
Injection buffer	86	8.1
High calcium	34	5.9
Oocyte extract	40	17.5
Whole-body extract	17	5.9
Boiled-sperm extract	59	15.3
Protease-treated sperm extract	32	6.3
<10 kDa fraction of sperm extract	42	21.4
All controls (total N and avg. % oscillations)	310	11.9

[a] Number of oocytes injected; for high calcium injections, 17 oocytes were injected with excess calcium in the form of 50 μM $CaCl_2$, and 17 were injected with a Ca-EGTA buffer containing 40 μM free calcium; for protease-treated sperm extract, 12 oocytes were injected with protease-treated sperm extract without subsequent PMSF treatment, and 20 were injected with protease-treated sperm extract that had been subsequently deactivated with PMSF.

[b] Percentage of oocytes displaying at least two Ca^{2+} transients within 30 min after injection; all values are significantly less at the $P = 0.005$ level than the 90.5% obtained for 180 oocytes injected with sperm extract.

lar concentrations attained following dilution with fluorescent dyes and injection, virtually all oocytes (16/18) given the extract externally failed to display Ca^{2+} oscillations (Figs. 3G and 9A). Moreover, oscillations were also absent in another 11 of 11 cases where the micropipette was placed on dye-loaded cells and repeated blasts of undiluted sperm extract were directed over oocytes in seawater containing 5% sperm extract, which in turn argues against the notion that intracellular injections simply delivered concentrated sperm extract to oolemmal surface receptors prior to introducing the injectate.

In addition, external applications of sperm extract did not trigger maturation in any of the 29 specimens imaged by confocal microscopy or in virtually any of the nonimaged specimens reared in cultures (Fig. 9B). Accordingly, nearly all Hoechst-labeled specimens receiving externally applied sperm extract remained arrested at metaphase I with a single set of chromosomes rather than polar bodies (Fig. 7E). Alternatively, fertilized oocytes showed high rates of maturation compared to unfertilized controls or specimens receiving sperm extract externally (Fig. 10B), and 83 ± 11% of the fertilized controls in these cultures ($N = 3$) developed into normal blastulae, indicating that the lack of maturation observed with external applications of sperm factor was not simply due to specimen morbidity.

The Oscillogenic Activity of the Sperm Extract Is Heat- or Protease-Labile and Is Fully Retained in >10-kDa Fractions, but Not in <10-kDa Fractions

Freshly prepared sperm extract retained some oscillogenic activity for 2 hr at room temperature or after having been frozen at −80°C for a few days. However, the extract lost potency when held overnight in a refrigerator, suggesting the presence of a labile component. To determine if sperm extracts can withstand high temperatures, aliquots of freshly prepared extract were boiled for 10–60 min before being cooled and combined with fluorescent dyes. In response to injections of boiled sperm extracts, few oocytes showed Ca^{2+} oscillations or meiotic maturation (Table 2), but 20 of 25 nonoscillating oocytes that had been injected with boiled extract subsequently oscillated in response to insemination, indicating that the boiled extract does not prevent oscillations (Fig. 10A).

A similar lack of oscillations was observed if, instead of boiling, the sperm extract was mixed with 0.03% protease prior to injection (Table 2). However, the viability of these control oocytes was questionable, since 0 of 12 specimens tested showed oscillations after sperm was added. Thus, protease-treated sperm extract was also deactivated with PMSF prior to injection. In 18 of 20 oocytes given such treatments, no oscillations occurred before fertilization (Table 2), but in 17 of 20 cases, repetitive Ca^{2+} waves were apparent after insemination (Fig. 10B). Conversely, if just PMSF without protease was added to the sperm extract before injection, Ca^{2+} oscillations occurred in all 18 unfertilized specimens examined (Fig. 10B, inset), indicating that protease rather than PMSF eliminated extract-induced oscillations.

Full oscillogenic activity was also retained in the relatively high-molecular-weight separations of the sperm extract, as intracellular injections triggered repetitive Ca^{2+} waves in 94.4% of the 18 specimens injected with the >100-kDa fraction and in 95.4% of the 44 specimens injected with the 10- to 100-kDa fraction (Figs. 3C and 11A). Oocytes injected with the <10-kDa fraction, however, typically failed to show calcium oscillations unless sperm was subsequently added to the specimen dish (Table 2; Fig. 11B).

DISCUSSION

Injections of Sperm Extracts Suggest the Presence of a Soluble Oscillogen in C. lacteus Sperm

Sperm contact hypotheses postulate that the binding of sperm to oolemmal receptors plays a key role in triggering the activating calcium transients of normal fertilization. However, in this study, repetitive Ca^{2+} oscillations and meiotic maturation are consistently elicited in unfertilized oocytes that were injected with sperm extract, even though interactions between whole sperm and oolemmal receptors are circumvented by such intracellular injections. The spatiotemporal properties of these Ca^{2+} oscillations, as well as the high rate of meiotic maturation induced by sperm ex-

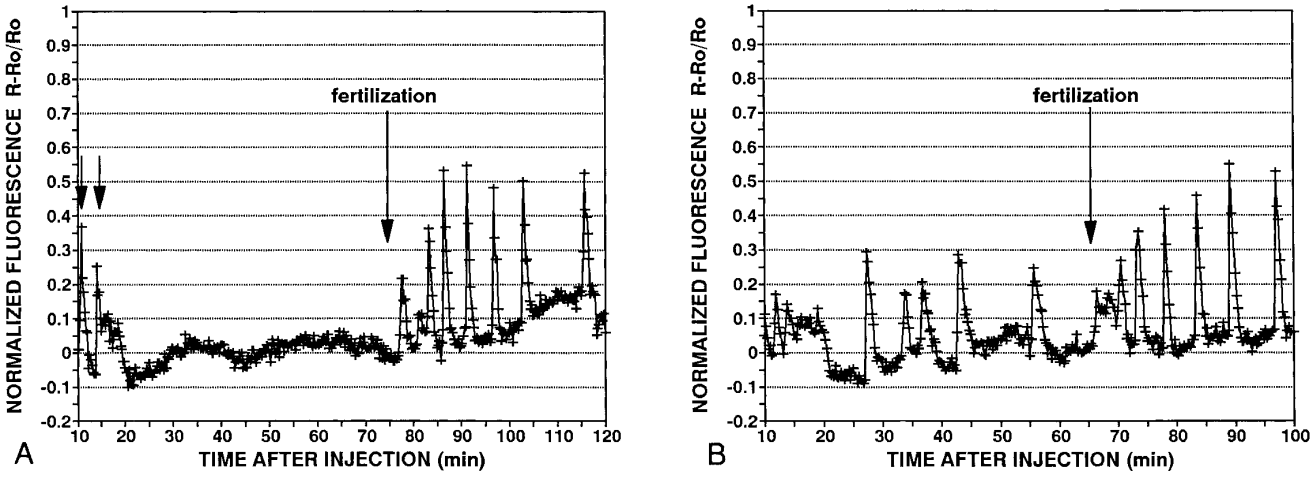

FIG. 5. Abnormal Ca^{2+} oscillations triggered by control injections. In controls that show two or more calcium transients by 30 min postinjection, the multiple calcium waves are sometimes ephemeral, irregular, and/or low in amplitude compared to those induced by fertilization or sperm-extract injections. Thus, the percentages of oscillating controls listed in Table 2 provide a conservative comparison between specimens injected with sperm extract and controls that truly mimic the calcium response elicited by sperm extract injections. (A) Two prefertilization Ca^{2+} transients (arrows) in a control oocyte injected with oocyte extract. (B) Irregular prefertilization oscillations in an oocyte injected with the <10-kDa fraction of sperm extract. R_0, initial CG/Rh ratio; R, subsequent CG/Rh ratios.

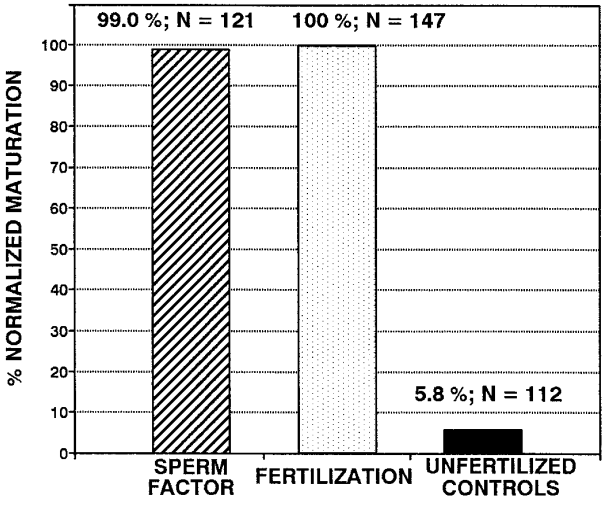

FIG. 6. Sperm factor injections are as effective as fertilization in triggering maturation. About 1–2 hr after either injection or fertilization, specimens were scored for maturation by checking for polar body formation, and the percentage of maturation was normalized relative to the rate displayed in 147 oocytes at 1–2 hr postfertilization (29.2% of the 147 fertilized oocytes had clearly observable polar bodies that were not obscured by under- or overlying ooplasm, and their overall maturation percentage was at least 80% based on subsequent cleavage rates). Such analyses show that maturation routinely occurred in 121 unfertilized oocytes that were injected with the soluble sperm extract, whereas virtually no maturation was evident in 112 unfertilized control oocytes obtained in essentially equal numbers from the seven categories listed in Table 2. *N*, number of oocytes examined.

tract injections, are closely reminiscent of the patterns observed in normal fertilizations. Conversely, similar signs of egg activation are typically lacking in numerous controls tested, even though such controls are clearly capable of producing Ca^{2+} oscillations in response to subsequent fertilizations. Collectively, such findings reveal that in *C. lacteus* the binding of sperm to oolemmal receptors is not absolutely necessary for triggering Ca^{2+} oscillations and meiotic maturation in unfertilized oocytes, and as alternative functions, such sperm-binding receptors may serve to (i) promote species-specific binding of gametes, (ii) aid in the attachment or incorporation of sperm; and/or (iii) initiate the block to polyspermy. However, pipet penetrations during sperm extract injections may artificially substitute for an early part of the calcium response (e.g., the ''cortical flash'' of external calcium influx) that is normally elicited by sperm binding (Stricker, 1996). Thus, whether oolemmal receptors are actually required for a calcium response during fertilization remains unclear.

The failure of excess-calcium injections to elicit Ca^{2+} oscillations or maturation also argues against a basic tenet of calcium bomb hypotheses and in turn supports the conclusions of Dale *et al.* (1985) that calcium ions by themselves cannot account for the activation elicited by sperm extract injections in sea urchin eggs. However, a possible problem with the high-calcium buffers used in this study is that the type of chelating solution can greatly influence the efficacy of calcium injections (Swann and Whitaker, 1986), and the calcium green probe added to these buffers has the confounding effect of binding calcium ions. Thus, such mixtures may not fully mimic the modes of calcium loading proposed in calcium bomb hypotheses. Neverthe-

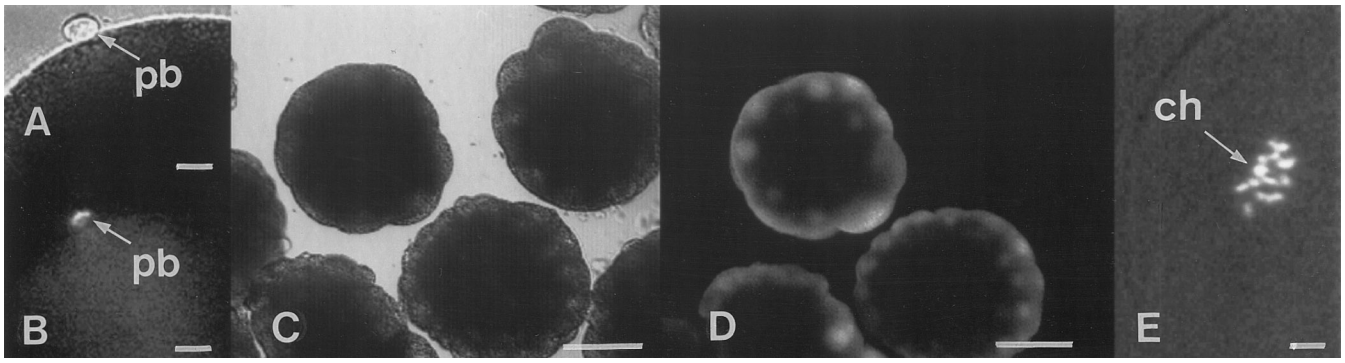

FIG. 7. Maturation and development. Bright-field (A) and correlative UV fluorescence image (B) of a Hoechst-labeled unfertilized oocyte that received a sperm extract injection and formed a polar body (pb) by 50 min postinjection. Bright-field (C) and correlative confocal fluorescence image (D) of three control specimens that had been injected with fluorescent dyes and subsequently fertilized after failing to show Ca^{2+} oscillations prior to insemination. Like neighboring non-dye-loaded specimens, such controls developed into blastulae, indicating that the lack of oscillations following control injections was not simply due to specimen morbidity. (E) UV fluorescence image combined with low-level bright-field illumination indicating that exogenously applied sperm extract does not trigger maturation, since chromosomes (ch) are still present at the metaphase-I plate in a Hoechst-labeled specimen that had been incubated for 2 hr in seawater containing 5% sperm extract. Scale bar, 10 μm for A, B, and E; 50 μm for C and D.

less, excess calcium probably does not trigger Ca^{2+} oscillations by itself, given that (i) aging, boiling, or protease digestions should not markedly affect Ca^{2+} concentrations and yet such treatments essentially abolish sperm extract activity; (ii) exactly opposite to what would be expected if calcium ions played a key role, the >10-kDa fractions of the sperm extract, but not the <10-kDa fractions, retain full oscillogenic activity; and (iii) ryanodine, IP_3, or excess KCl typically causes a single Ca^{2+} transient in unfertilized oocytes, but such individual transients do not lead to Ca^{2+} oscillations (Stricker, 1996).

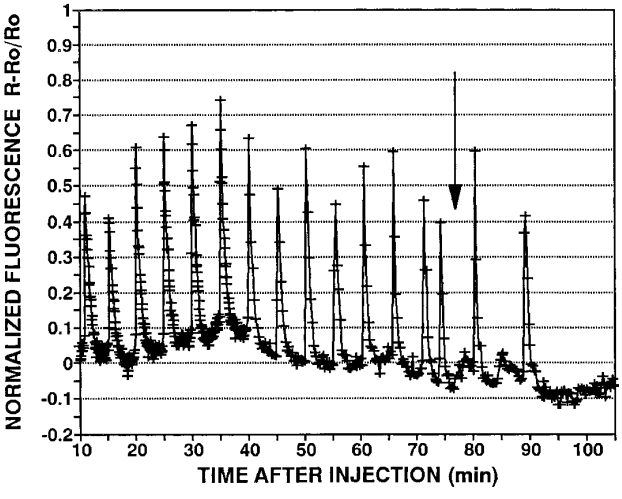

FIG. 8. Insemination of sperm-extract-injected oocytes does not noticeably prolong or alter extract-induced Ca^{2+} oscillations. Calcium trace of an unfertilized oocyte that had been injected with sperm extract and then inseminated at 77 min postinjection (arrow), suggesting that sperm extract injection may help to provide a block to subsequent sperm fusion/incorporation. R_0, initial CG/Rh ratio; R, subsequent CG/Rh ratios.

As an alternative explanation for egg activation, the sperm oscillogen model (Swann, 1993, 1996) makes several predictions, which coincide well with the results obtained in this study (Fig. 12). First, intracellular injections of sperm extracts, but not control solutions, consistently induce Ca^{2+} oscillations as well as maturation in unfertilized oocytes of *C. lacteus.* The fact that the extract routinely elicits maturation is particularly noteworthy, considering that only fertilization, and not treatments with IP_3, ryanodine, or excess K^+, triggers maturation in this species (Stricker, 1996). In addition, the extraction techniques do not yield oscillogenic extracts regardless of the source of cells used, since extracts of oocytes or whole pieces of unripe males do not cause high rates of oscillations or maturation. Such findings indicate that the oscillogen is relatively concentrated in sperm and/or more readily extracted from sperm than from the other cells tested.

Although these results support a sperm content type of hypothesis, they do not rule out sperm contact hypotheses, not only for reasons discussed above regarding pipet impalements, but also because of the possibility that multiple signaling pathways exist during fertilization. Thus, oolemmal receptors may not be required for the activating calcium response when injecting sperm extracts. However, during normal fertilization, sperm–receptor binding might stimulate a redundant pathway for generating Ca^{2+} transients, in a manner analogous to what has been maintained for sea urchin eggs by some (Galione *et al.*, 1993; Lee *et al.*, 1993), but not all (Mohri *et al.*, 1995; Lee *et al.*, 1996), investigators. Alternatively, oolemmal receptors and sperm factors could act together such that the binding of sperm primes the egg for the actions of sperm factors, which in

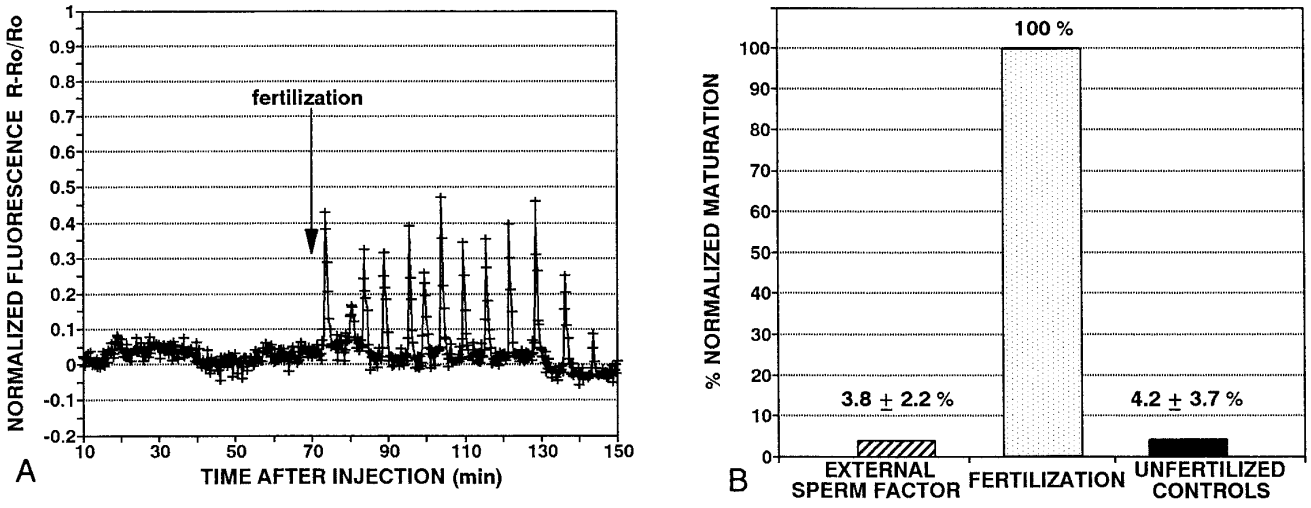

FIG. 9. External applications of sperm extract are ineffective in triggering Ca^{2+} oscillations or maturation. (A) Calcium trace of an oocyte injected with fluorescent dyes and treated at ~12 min after the dye injection with 5% sperm factor in the surrounding seawater (i.e., extract made from an $\sim 1.5 \times 10^9$ sperm/ml mixture was diluted 1:19 with seawater to yield a final 5% concentration that in turn doubles the 1–2.5% intracellular concentrations normally attained after extract injections). In the presence of externally applied sperm factor, Ca^{2+} oscillations are lacking until sperm is added to the specimen dish (~70 min after the dye injection). (B) About 1–2 hr after the onset of each of three experiments: (i) specimens were fixed and scored for maturation by checking for polar body formation, and (ii) the percentage of maturation was normalized relative to the rate displayed in fertilized oocytes (25.7 ± 4.3% of the fertilized oocytes in three cultures had clearly observable polar bodies that were not obscured by under- or overlying ooplasm, and their overall maturation percentage was at least 80% based on subsequent cleavage rates). Such analyses indicate that unlike the maturation routinely elicited by fertilization, low rates of maturation occur in cultures of unfertilized oocytes that were incubated at 12°C in seawater containing (i) 5% sperm extract (external sperm factor) or (ii) 5% injection buffer without sperm extract (unfertilized controls). R_0, initial CG/Rh ratio; R, subsequent CG/Rh ratios.

turn might mean that abnormally high amounts of sperm extract are needed to activate eggs in the absence of sperm–receptor binding.

Accordingly, as noted previously (Foltz and Shilling, 1993; De Nadai *et al.*, 1996; Xu *et al.*, 1996) receptor-mediated and oscillogen-induced modes of Ca^{2+} mobilization need not be mutually exclusive, if surface receptors and sperm factors function redundantly or synergistically during fertilization. To assess these possibilities, specific means of inhibiting the activities of oolemmal receptors vs sperm factors are needed so that selective eliminations of functionality during fertilization can be analyzed. Moreover, data obtained from one model system should not necessarily be extrapolated to other animal groups, since, as noted by Foltz and Shilling (1993), significant species-specific differences may exist in fertilization-induced Ca^{2+} signaling.

The Oscillogenic Component of the Sperm Extract Appears to Be a Protein, Such as the 33-kDa Oscillin Protein of Mammalian Sperm

Based on the fact that sperm extracts of *C. lacteus* lose activity following aging, boiling, or protease treatment, the oscillogenic component of the extract is apparently a protein, as postulated in the sperm oscillogen hypothesis (Swann, 1993, 1996). Moreover, impermeant proteins should be ineffective in activating eggs when applied to the exterior of the oolemma. Accordingly, only intracellular injections, and not external applications, of the *C. lacteus* sperm extract trigger Ca^{2+} oscillations and meiotic maturation.

Results obtained from fractionations of the crude extract also indicate that the activity of the sperm extract is due to a protein (or at least some macromolecule), because the >10-kDa fractions retain full activity, whereas the <10-kDa fractions are not markedly active. Although the 21% rate of oscillating oocytes obtained after injecting the <10-kDa fraction is somewhat higher than that observed in other controls, the slightly elevated activity could simply be due to a minor leakage of >10-kDa molecules across the filter (Amicon Corp. Instruction Booklet). In any case, the number of oscillating specimens occurring in response to injections of the <10-kDa fraction is significantly lower than that obtained with the >10-kDa fractions, which in turn would not be expected, if in fact the oscillogenic component were a small, nonproteinaceous factor such as calcium ions (Jaffe, 1991) or IP_3 (Tosti *et al.*, 1993).

Injections of sperm extracts yield calcium responses in various mammalian oocytes (Stice and Robl, 1990; Swann, 1990; Swann and Ozil, 1994; Homa and Swann, 1994; Dale *et al.*, 1996; Wu *et al.*, 1997), and in the case of hamster

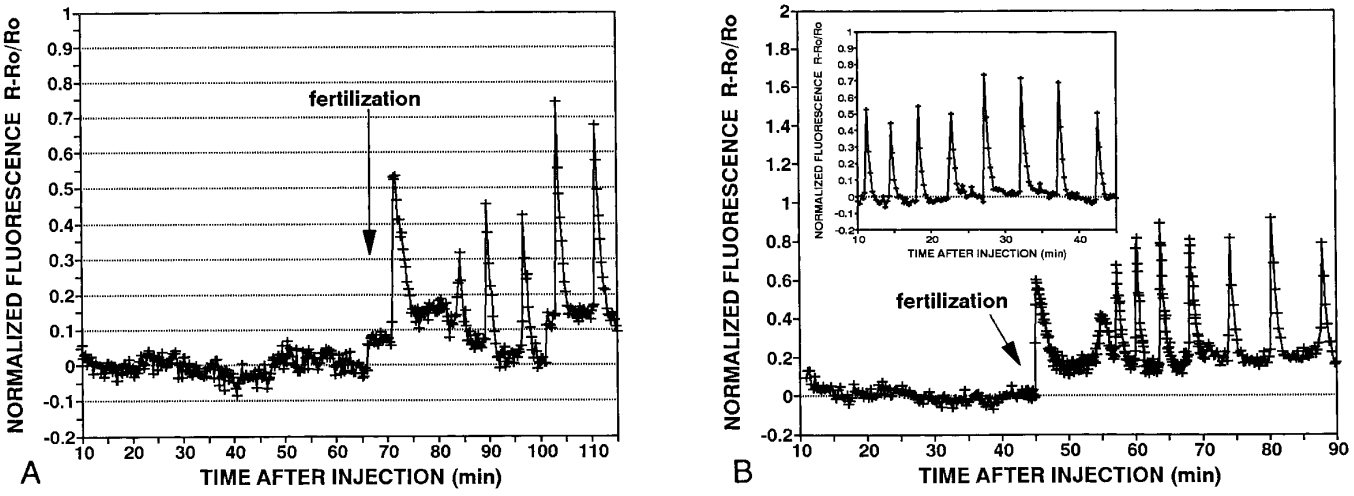

FIG. 10. Boiling or protease treatment essentially abolishes sperm extract activity. (A) Following injection of boiled sperm extract, Ca^{2+} oscillations are lacking for 50 min of imaging, but are evident after sperm is added to the specimen dish (~65 min postinjection). (B) After treating sperm extract with 0.03% protease and subsequently deactivating the protease with PMSF, no prefertilization Ca^{2+} oscillations are evident for 35 min of imaging, even though the oocyte is capable of producing oscillations upon subsequent fertilization at 45 min postinjection. (Inset) Injection of sperm extract treated with PMSF but not protease causes oscillations, indicating that protease and not PMSF eliminates the oscillogenic activity of the extract. R_0, initial CG/Rh ratio; R, subsequent CG/Rh ratios.

sperm extracts, the active component has been identified as a 33-kDa protein, termed oscillin (Parrington *et al.*, 1996). The underlying reasons for the positive effects of both the 10- to 100-kDa and >100-kDa fractions of the *C. lacteus* sperm extract remain unknown. Perhaps, as described for oscillin (Parrington *et al.*, 1996), >100-kDa oligomers of the oscillogenic protein have biological activity. Alternatively, the relatively short centrifugations used in the separation protocol could have allowed the C-100 retentate to be contaminated with <100-kDa molecules. In any case, the results obtained with protease treatments and the >10-kDa fractions of *C. lacteus* sperm extract tentatively suggest that this species may also contain an oscillin-like protein, but further investigations are certainly needed to determine

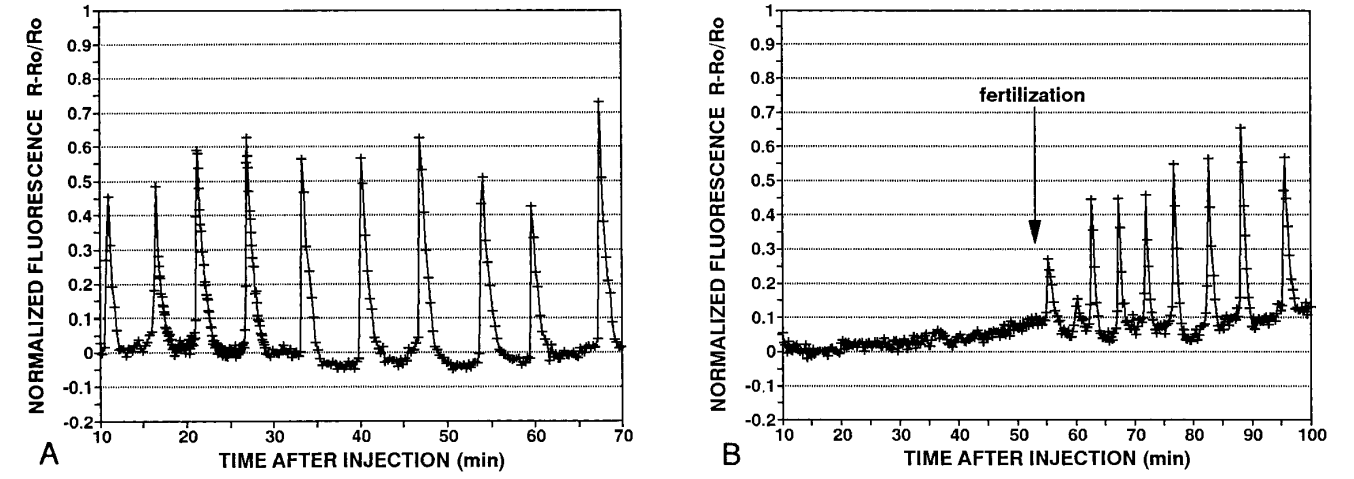

FIG. 11. Oscillogenic activity is fully retained in >10-kDa fractions of the sperm extract, but not in <10-kDa fractions. After partitioning the crude sperm extract by means of molecular-weight-cutoff filters, injection of the 10- to 100-kDa fraction (A) causes Ca^{2+} oscillations. Conversely, injection of the <10-kDa fraction (B) does not tend to trigger oscillations, even though such control oocytes can oscillate following addition of sperm to the specimen dish (in this case, at ~50 min postinjection). R_0, initial CG/Rh ratio; R, subsequent CG/Rh ratios.

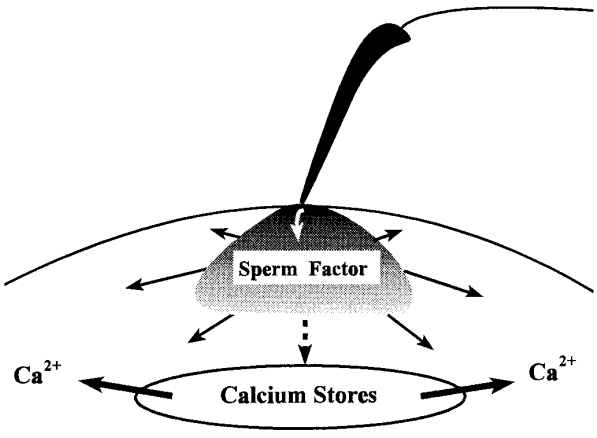

FIG. 12. The sperm oscillogen model of signal transduction (Swann, 1996): A proteinaceous sperm factor is introduced inside the oocyte to trigger Ca^{2+} oscillations and egg activation via the release of internal stores of bound calcium. Supporting data from analyses of *C. lacteus* include (i) intracellular injections of sperm extract bypass interactions between whole sperm and oolemmal surface receptors but nevertheless routinely cause Ca^{2+} oscillations and meiotic maturation in unfertilized oocytes of *C. lacteus;* (ii) egg activation is not simply due to the injection procedure, since various control injections, including (a) injection buffer alone, (b) buffer with excess Ca^{2+}, or (c) extracts made from cells other than sperm, typically fail to induce Ca^{2+} oscillations and maturation, even though such controls are routinely activated upon subsequent addition of sperm; (iii) the spatiotemporal properties of the Ca^{2+} oscillations triggered by sperm extract injections closely resemble those elicited by fertilization or whole sperm injections; (iv) sperm-extract-induced oscillations persist in calcium-free seawater; (v) external applications of sperm extract are ineffective in triggering oscillations or maturation; (vi) sperm extract activity is heat- or protease-labile; and (vii) full oscillogenic activity resides in >10-kDa fractions, but not in <10-kDa fractions, of the sperm extract.

whether the oscillogenic activity of *C. lacteus* sperm extracts is in fact due to oscillin or to some other molecule.

Does a Single Sperm Contain Enough Oscillogen to Activate an Egg?

Data presented in this study do not prove that a single sperm has sufficient oscillogen to activate eggs. However, as the following calculations indicate, Ca^{2+} oscillations are elicited after injecting only a few ''sperm equivalents'' of extract, and a single sperm may indeed have enough oscillogen for full egg activation, if sperm-equivalent calculations are adjusted for inefficient extractions and/or losses of oscillogenic activity following extraction.

Based on an average of 1.5×10^9 sperm/ml in each extract, 1 pl of extract theoretically contains 1.5 sperm equivalents of oscillogen, if the extraction procedure achieved a 100% yield of active oscillogen from each sperm. Uncompressed oocytes typically range in size from 100 to 130 μm (Stricker, 1996). Thus, assuming a spheroidal shape and a midrange diameter of 115 μm, the oocyte has a calculated volume of 796 pl. Since injection volumes constituted 2–5% of the oocyte volume, an average injection of 3.5% contains 0.035 oocyte volume/injection (×) 796 pl/oocyte volume (×) 1.5 sperm equivalents/pl = 41.8 sperm equivalents/injection, if pure extracts were injected. However, since the extract was diluted 1:1 with fluorescent dyes prior to injection, 20.9 sperm equivalents of oscillogen are delivered in an average injectate that has ample capacity to activate eggs. Alternatively, since such numbers calculate the average sperm equivalents eliciting full activation in >90% of the specimens tested, some eggs can theoretically be activated by values approaching the 5.3 sperm equivalents of extract that is calculated when using the lower end of each data range (i.e., sperm concentration, 1×10^9/ml; injection volume, 2%; and oocyte volume, 524 pl).

Injecting a 10-fold dilution of the 1.5×10^9 sperm/ml extract to deliver 2.1 sperm equivalents can still generate Ca^{2+} oscillations, although such repetitive transients have a reduced frequency and an undetermined ability to activate eggs. A 30-fold dilution that provides only 0.7 sperm equivalents, on the other hand, fails to elicit a calcium response. Thus, (i) injecting an average of 21 sperm equivalents of extract triggers complete egg activation in >90% of the oocytes tested, (ii) some oscillations occur with injections of 2.1 sperm equivalents, and (iii) 0.7 sperm equivalents of extract are unable to generate a calcium response. Collectively, such data indicate that the injection of as little as 2.1 sperm equivalents of extract can generate Ca^{2+} oscillations, and the minimum amount of oscillogen needed for full activation is apparently present in 0.7–21 sperm equivalents of the extract.

It is important to note, however, that sperm equivalent units cited above are calculated based on all of the active oscillogen being obtained from each sperm, and as such may significantly exaggerate the number of sperm that are actually required to provide an egg-activating extract. This is because such calculations assume (i) the sperm oscillogen is completely solubilized during extraction and (ii) the soluble oscillogen retains full activity prior to injection. However, given some incompletely disrupted sperm in the extracted sperm pellet (unpub. obs.), it seems highly likely that <100% of the oscillogen was solubilized. Moreover, based on the demonstrated heat- and protease-lability of the sperm extract (Fig. 10; Table 2), the solubilized oscillogen presumably lost activity prior to injection, especially considering that disrupted acrosomal vesicles would undoubtedly release proteases, and protease inhibitors were not added to the extraction buffer. If adjustments are made for incomplete extractions and losses of activity prior to injection, the 0.7–21 sperm equivalent range could be greatly lowered and hence more supportive of the view that a single sperm contains enough oscillogen for egg activation. In order to assess exactly how much nonadjusted calculations overestimate sperm equivalents levels, the actual efficiencies of solubilization and activity retention must be ascertained, and such processes cannot be quantified until the

oscillogen is identified and its active concentration is reliably measured.

In accordance with the sperm extract data, intracellular injections of whole *C. lacteus* sperm can induce Ca^{2+} oscillations with spatiotemporal properties closely resembling those of normal fertilization (Stricker, 1996). Such repetitive Ca^{2+} waves are not associated with maturation in the few cases tested (Stricker, 1996), but this could simply be due to the deleterious effects of injecting a relatively large (15-μm-long) sperm without the proper injection buffer or delivery techniques needed to sustain normal development. In humans, where the method of intracytoplasmic sperm injection (''ICSI'') has been perfected for routine use in fertility clinics, injection of either sperm extract (Homa and Swann, 1994) or a single whole sperm (Tesarik and Souza, 1994) produces Ca^{2+} oscillations, and the ICSI procedure clearly yields viable offspring (Harari *et al.*, 1995). Collectively, such findings indicate that an internally delivered sperm with its putative complement of sperm factor and other vital components is capable of promoting normal development.

Individually, none of these calculations or observations confirms that the putative oscillogen of *C. lacteus* is sufficiently concentrated in a single sperm to play a biological role during fertilization. However, the findings also do not rule out this possibility, since an extensive dilution series was not tested to determine if the extract concentration used in this study could be significantly reduced while still maintaining oscillogenic and activating capabilities. In order to ascertain the minimum amount of oscillogen needed for egg activation more accurately than the range of values reported in this study, precisely quantified injections are needed. More importantly, sperm-equivalent calculations must be rigorously assessed and not simply based on sperm counts, injection volumes, and an assumption that the extraction procedure provides a 100% yield of active oscillogen.

Calcium Oscillations and Egg Activation in C. lacteus

The fact that the Ca^{2+} oscillations induced by fertilization or sperm-extract injections continue to occur in CaFSW suggests that the repetitive calcium waves are dependent upon internal Ca^{2+} release rather than external Ca^{2+} influx. However, the exact means by which the release of internal calcium stores is modulated remains unknown. In mammalian oocytes, the oscillogenic protein oscillin is thought to generate oscillations by directly affecting IP_3 and/or ryanodine types of calcium-channel receptors (Berridge, 1993; Sorrentino, 1995) associated with internal calcium stores, rather than by causing oscillating fluxes in activators of these receptors (Swann, 1996). This conclusion is based on the fact that calcium, IP_3, cyclic ADP-ribose, or other modulators of calcium mobilization fail to trigger fertilization-like Ca^{2+} oscillations, but such oscillations are consistently generated by sperm extracts or the thiol-reducing agent thimerosal, which in turn is believed to sensitize channel receptors (Swann, 1996). Currently, there is no evidence to distinguish whether the putative *C. lacteus* oscillogen directly affects ooplasmic Ca^{2+} channel receptors associated with internal stores of bound calcium or otherwise causes oscillations in upstream modulators of these stores.

Regardless of how they are produced, Ca^{2+} oscillations seem to provide a signal for maturation, given that (i) oocytes that generate a single Ca^{2+} transient do not mature, (ii) maturation consistently proceeds in oscillating specimens that had been either fertilized or injected with sperm extract, and (iii) in the few aberrant control oocytes that do mature, spontaneous oscillations occur prior to maturation. Similarly, although mammalian oocytes can be activated by a single Ca^{2+} transient (Swann and Ozil, 1994; Kline, 1996), there is mounting evidence that repetitive oscillations are more effective triggers of activation (Ozil and Swann, 1995). Such findings suggest the release from metaphase-I arrest and the subsequent stages of maturation in *C. lacteus* are triggered by frequency-encoded information delivered by Ca^{2+} oscillations but not by a single Ca^{2+} transient (Tang and Othmer, 1995).

The potential importance of oscillation frequencies is further underlined by recent findings that altering oscillation patterns during the first cell cycle in mouse embryos can lead to variations in the cellular composition of the blastocyst that develops, indicating possible long-term consequences of Ca^{2+} oscillations during mammalian embryogenesis (Bos-Mikich *et al.*, 1997). Moreover, the injection of a sperm extract from pigs elicits Ca^{2+} oscillations in both cow and mouse oocytes (Wu *et al.*, 1997). However, the frequencies of the oscillations induced by the heterologous extract differ significantly in the cow vs mouse and match the frequencies characteristically elicited by fertilization in these two species (Wu *et al.*, 1997). Thus, there may be species-specific differences in oscillation frequencies that serve to promote normal development.

Clearly, several key aspects of fertilization-induced Ca^{2+} dynamics in *C. lacteus* remain poorly understood. For example, the exact nature of the putative oscillogen, its concentration within individual sperm, and the mechanisms by which it might elicit repetitive Ca^{2+} waves are unknown at this point. Moreover, whether normal fertilization actually utilizes the signaling pathway triggered by injections of sperm extract remains untested. Nevertheless, this investigation provides new evidence supporting the sperm oscillogen model of signal transduction that has been proposed based on studies of mammalian fertilization (Swann, 1996). The demonstration of such patterns in a group that differs from mammals in both its fertilization biology and phylogenetic position (Brusca and Brusca, 1990; Turbeville *et al.*, 1992; Winnepenninckz *et al.*, 1995) raises the question of how common it is within the animal kingdom for soluble sperm proteins to elicit calcium responses and egg activation, particularly in oocytes that generate Ca^{2+} oscillations rather than a solitary Ca^{2+} wave.

ACKNOWLEDGMENTS

I thank L. Hertel and E. Loker for providing valuable advice and for generously supplying equipment. D. Battaglia, D. Epel, H.

Othmer, and K. Swann also made many helpful comments. This study was supported in part by NSF Grant 92-53052.

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Received for publication November 19, 1996
Accepted April 14, 1997